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A MORPHOLOGICAL ASSESSMENT OF THE STIMULATORY EFFECT OF COUMARIN ON MACROPHAGES

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Summary.—In examining the migration of macrophages on to subcutaneously implanted coverslips in rats, it was found that coumarin (5–6 benzo-α-pyrone) and a preparation (Venalot®) containing the former, very signficantly increased the total macrophage number as well as the percentage of stimulation on the skin side of the coverslip. For the muscle side, Venalot increased the total numbers and the percentage stimulated while coumarin alone had no effect compared with the control. It remains to be seen whether these stimulated macrophages are also activated.

BENZOPYRONES (which include coumarin) have, at various times by various methods, been shown to "stimulate" macrophages. Kovach *et al.* (1965) showed coumarin to enhance carbon clearance from the blood. Piller (1976*a*) found evidence which suggested there was an increase in the number of phagocytosing sites. Bolton and Casley-Smith (1975), Piller (1977) and Dunn *et al.* (1977) have all shown an enhanced phagocytosis by macrophages following coumarin administration. Piller (1976*b*, *c*) has shown an elevation of macrophage enzyme activity levels and digestion products in the extracellular environment. While it seems sure that the benzopyrones (in particular coumarin) can "stimulate" macrophages, there are a number of important criteria (Evans, 1972; Alexander, 1976) which have not been examined in detail. In this light, the ability of macrophages to spread on to a glass coverslip and the number which are in a "stimulated" state as assessed by morphological criteria has been investigated.

MATERIALS AND METHODS

Thirty female albino Sprague–Dawley rats (200 ± 20 g) were randomly divided into three equal groups. One group received 25 mg/kg coumarin (5-6-benzo-α-pyrone) in 2% A.R. ethanol in physiological saline, while the other received Venalot® (Schaper and Brümmer, West Germany). Each millilitre of this preparation contains 1·5 mg coumarin and 25 mg rutin sodium sulphate salts. It was administered so that the daily dose of coumarin was the same as the group which received this only. The third group served as a control and was given an equivalent volume of 2% A.R. ethanol in physiological saline. All injections were i.p.

After two daily injections, the rats were anaesthetized with an i.p. injection of 0·75 ml of a 10% solution of Sagatal (May and Baker, England) in 10% A.R. ethanol/100 g body wt.

Both flanks of each rat were closely shaved and a small lateral incision made at about the region of the last rib. The skin was carefully separated from the tight fascia immediately overlying the muscle. The cut and the region of separation were just large enough to insert two 18 mm coverslips back to back on each flank. Insertion was in the anterior direction. The procedure was carried out under sterile conditions with sterile polished round coverslips (Menzel Gläser, West Germany). All air was gently forced from the pocket and the wound sewn.

Injections were continued for a further 2 days. Two hours after the last injection, the coverslips were removed (total *in situ* time of 50 h), washed in physiological saline to remove non-adherent cells, then stained with May–Grünwald solution (Merck) and counterstained with Giemsa solution (Merck). The coverslips were cemented to glass slides and the macrophage numbers assessed.

For this, an ocular grid was first used combined with a low-power objective. Each square of the grid was assigned a number and then with random number tables a given square was chosen. Five fields in the square were examined in each of 10 squares using a Kp1w10X/20 eyepiece (Zeiss, West Germany) and a planapo 63/1·4 oil 160/objective (Zeiss). For each field, the total number of macrophages and the number "stimulated" were recorded. Fields near the edge of the coverslips were ignored.

To be classified as "stimulated" a macrophage had to possess two distinguishable pseudopodic extensions and at least 10 phagocytic vacuoles (photographs of Cohn and Benson, 1965). The latter were, however, difficult to distinguish at times, depending on the extent of the macrophage spreading.

RESULTS

The results are summarized in the figure.

Skin side

Control.—There was an average total number of 5 s.d.±0·62 macrophages per field of view, of which 13% (0·64, s.d.± 1·97) were stimulated.

Coumarin.—On average there was a total of 20±13·8 macrophages; this was very significantly ($P<0·001$) more than the control group. Of these, 48% (9·7, s.d. ±7·2) were stimulated, which was also very significantly higher ($P<0·001$) than the control.

Venalot.—On average there was a total of 13±7·4 macrophages. This was also very significantly higher ($P<0·001$) than the control. Of these, 87% (11·2±7·7) were stimulated ($P<0·001$).

Muscle side

Control.—There was an average of 5·4±13·5 macrophages, of which 30% (1·6±4·7) were stimulated.

Coumarin.—On average there were 1·7 ±2·8 macrophages; of these, 80% (1·4± 2·3) were stimulated. There was no significant difference between these results and those of the control.

Venalot.—On average there were 11·5± 7·2 macrophages; of these, 84% (9·7±6·1) were stimulated. In both cases this was very significantly greater ($P<0·001$) than the control.

Skin–muscle comparison

Of the respective groups, only the coumarin-treated group showed any signi-

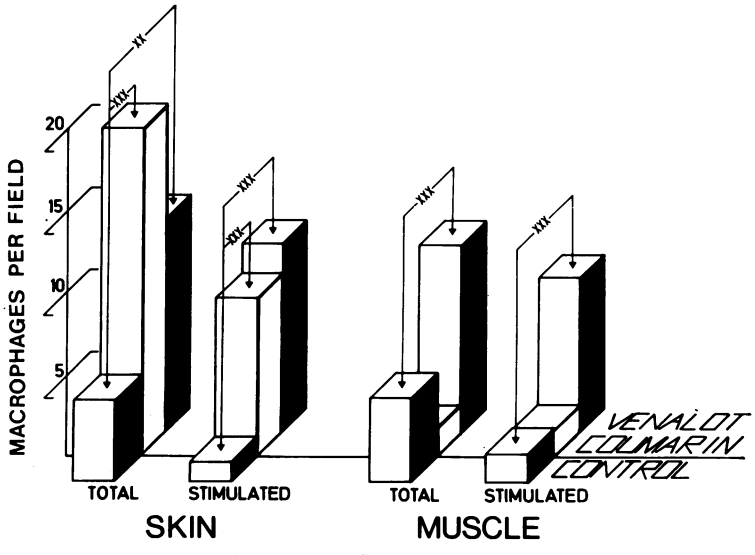

FIG.—Total macrophage numbers and proportion stimulated per high power field of view. Each observation is the mean of 5 fields in each of 10 randomly chosen squares on each coverslip in 10 rats.

ficant change in the total macrophage number and percentage stimulation. In both cases (total numbers and proportion stimulated) it involved a very significant reduction ($P<0{\cdot}001$) on the muscle side.

DISCUSSION

The stimulation of macrophages by benzopyrones, including coumarin, is well documented (for review see Piller, 1977). In this report, coumarin and Venalot have both been shown to increase not only the total numbers of macrophages migrating on to subcutaneously planted coverslips, but also the percentage which were determined (using morphological characteristics) to be stimulated. An exception was coumarin for the muscle side of the coverslips, where there was no difference compared with the control levels. However, the administration of Venalot (which contains rutin sodium sulphate salts in addition to coumarin) again gave significantly increased numbers of macrophages and percentage stimulation. Exactly why this occurs is not known.

These results have generally added weight to the other biochemical, morphological and cytochemical observations that coumarin and especially Venalot are stimulators of macrophages and in general the mononuclear phagocytic system (reviewed by Casley-Smith, 1976; Piller, 1977). Such an activity may be of some importance in the control of metastasis and destruction of tumour cells.

It is well known that the macrophage: tumour cell ratio is also very important as far as the ability of animal tumours to metastasize is concerned (Eccles and Alexander, 1974). Alexander (1976) has found an inverse correlation between the macrophage content and the ability to metastasize, suggesting that the latter function may be related to the capacity of the macrophages to kill the tumour cells as a result of cell-to-cell contact. Also, it is known that macrophages can kill tumour cells in a nonspecific manner which is not dependent on any immunologically specfiic interaction between the macrophage and membrane antigens (Evans and Alexander, 1976).

It may be that these macrophages which show all the cardinal signs of "stimulation" upon coumarin administration may also be "activated" according to the terminology used by Alexander (1976) and Mauel (1976), and thus are capable of entering the tumours not only in higher numbers but with a higher percentage in a "stimulated" and perhaps an "activated" condition. Once there, a nonspecific destruction of the tumour cells may occur.

Work is currently in progress to determine the effect of coumarin on the macrophage: tumour cell ratio in solid tumours, whether there is an enhanced nonspecific destruction of the tumour cells and whether there is any effect on the ability of the tumour to metastasize.

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